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Early recruitment of natural CD4⁺ Foxp3⁺ Treg cells by infective larvae determines the outcome of filarial infection

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Human helminth infections are synonymous with impaired immune responsiveness indicating suppression of host immunity. Using a permissive murine model of filariasis, *Litomosoides sigmodontis* infection of inbred mice, we demonstrate rapid recruitment and increased *in vivo* proliferation of CD4⁺ Foxp3⁺ Treg cells upon exposure to infective L3 larvae. Within 7 days post-infection this resulted in an increased percentage of CD4⁺ T cells at the infection site expressing Foxp3. Antibody-mediated depletion of CD25⁺ cells prior to infection to remove pre-existing 'natural' CD4⁺ CD25⁺ Foxp3⁺ Treg cells, while not affecting initial larval establishment, significantly reduced the number of adult parasites recovered 60 days post-infection. Anti-CD25 pre-treatment also impaired the fecundity of the surviving female parasites, which had reduced numbers of healthy eggs and microfilaria within their uteri, translating to a reduced level of blood microfilaraemia. Enhanced parasite killing was associated with augmented *in vitro* production of antigen-specific IL-4, IL-5, IL-13 and IL-10. Thus, upon infection filarial larvae rapidly provoke a CD4⁺ Foxp3⁺ Treg-cell response, biasing the initial CD4⁺ T-cell response towards a regulatory phenotype. These CD4⁺ Foxp3⁺ Treg cells are predominantly recruited from the 'natural' regulatory pool and act to inhibit protective immunity over the full course of infection.

Key words: Parasitic helminth • Suppression • T cells



Supporting Information available online

Introduction

Chronic helminth infections are associated with impaired immune responsiveness [1–4], and helminth parasites have been widely attributed with the ability to manipulate and down-modulate protective immune responses [5–7]. During human filarial infec-

tions down-regulation is mainly characterized by a loss of antigen-specific proliferation together with impaired production of IFN- γ and IL-5 [8, 9], and IL-4 [10]. Regulatory roles have been ascribed to both TGF- β and IL-10 [10–12], two cytokines closely implicated in the activity [13, 14] and induction [13, 15–18] of Treg cells raising the hypothesis the Treg cells mediate helminth-induced immune suppression. Over the recent years, evidence has mounted for Treg activity not only in filarial infections [10, 19–23] but also among a range of other helminth pathogens including gut- and muscle-dwelling nematodes [24–27] and schistosomes [28–36].

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The major functions of Treg cells are to limit pathology by dampening immune inflammation, and more specifically to control autoimmunity [13, 37]. A repercussion of their immune down-regulatory function is that they can inhibit protective immune responses required to clear infection [38, 39]. Two main populations of Treg cells have been described. One subset are Tr1 cells defined by their production of IL-10 and considered to be 'adaptive' as they develop from naïve T cells in the periphery following antigen challenge [13]. The second population is of CD4⁺Foxp3⁺ Treg cells whose regulatory phenotype is driven by expression of the transcription factor Foxp3 [37, 40]. CD4⁺Foxp3⁺ Treg cells have two distinct origins. 'Natural' CD4⁺Foxp3⁺ Treg cells become committed to a regulatory phenotype within the thymus [41]. Foxp3 expression can, however, also be induced in naïve peripheral T cells [16–18], so that the CD4⁺Foxp3⁺ phenotype includes cells derived through both 'natural' and 'adaptive' pathways.

Both Tr1-like and Foxp3⁺ Treg cells are active during human filarial [10, 19–21], and *Schistosoma mansoni* infections [29, 30], suggesting that Tr1 and Foxp3⁺ Treg cells act in concert. It is not yet clear, however, what the respective roles of these cells are and whether Foxp3⁺ Treg cells found during human infections are of natural or adaptive origins. Studies in murine models of helminth infection have defined host-beneficial effects of Treg cells. Both Tr1-like cells [28] and CD4⁺CD25⁺Foxp3⁺ Treg cells play roles in controlling egg-induced pathology during *S. mansoni* infection [22, 31–36]. Strikingly, the increased Treg-cell activity seen during helminth infections can also down-regulate bystander inflammatory responses and can protect against allergic inflammation [26, 42, 43] and intestinal inflammatory disorders [27, 44].

The counter side is that while Treg cells may forestall pathogenesis, they can also inhibit protective immunity impeding effective parasite killing [39]. This trade-off is apparent during *S. mansoni* infection, in which depletion of CD25⁺ Treg cells increases liver pathology while reducing the host's egg burden, indicating that the beneficial anti-inflammatory role of Treg is at a cost to protective immunity [32]. The down-regulation of protective immunity may not just reflect a host compromise, however, and the presence of immunomodulatory Treg-cell population provides a potential Achilles heel for parasites to exploit. Helminth parasites produce homologues of mammalian TGF- β [45–47] of which at least one is able to bind human TGF- β receptors [46, 47] and could potentially directly drive a host Treg-cell response.

An important new model of immunoregulation in filariasis is *Litomosoides sigmodontis* infection of inbred mice, and we have previously characterized CD4⁺ T-cell responses in this system. BALB/c mice are susceptible to *L. sigmodontis*, developing a fully patent infection ~50 days post-infection, patency being defined as the presence of mature adult parasites within the pleural cavity and microfilariae (Mf, transmission stage L1 larvae) circulating within the blood [48, 49]. In contrast, resistant C57BL/6 mice clear their parasites between days 40 and 50 post-infection and never develop a patent infection. Notably, we found CD4⁺CD25⁺Foxp3⁺ Treg-cell activity in susceptible BALB/c mice during the

established adult stages (from 4 wk post-infection) of filarial infection [23, 50]. Moreover, during the adult stage the CD4⁺ effector T-cell (Teff cell) population at the infection site became unresponsive to antigen, which was associated with high levels of expression of both CTLA-4 and GITR. Once infection had established, parasite killing could be promoted by depleting CD25⁺ Treg cells, but only when performed in combination with an agonistic anti-GITR mAb to provide co-stimulation through GITR [50], or with a neutralising anti-CTLA-4 mAb to block co-inhibition through CTLA-4 [23]. This requirement for a dual-component treatment suggests that to disrupt established immune regulatory pathways depletion of CD25⁺ Treg cells alone is insufficient, and that the CD4⁺ Teff cell response must also be targeted and revitalized.

Interestingly, while Tr1 cells and IL-10 play regulatory roles in other helminth infections [19, 21, 28, 29, 35] neutralisation of the IL-10R or IL-10 deficiency fails to promote killing of *L. sigmodontis* parasites or restore T-cell responsiveness, except in the absence of IL-4 [50, 51]. Thus, in the presence of an intact Th2 response, immune-regulation during *L. sigmodontis* infection appears to be largely IL-10 independent with CD4⁺Foxp3⁺ Treg cells being the main active Treg cell inhibiting protective immunity. For the development of successful vaccines or therapeutic treatments to promote immunity or overcome regulation it is therefore important to determine the source of CD4⁺Foxp3⁺ Treg cells. Are they of 'natural' or 'adaptive' origins, and are they generated from the initial point of infection by infective-stage L3 larvae or arise subsequently as chronic adult worm infection reaches a more homeostatic state?

We now show that the infective L3 stage induce *in vivo* proliferation of CD4⁺Foxp3⁺ Treg cells, which within 7 days of infection translates to an increased percentage of CD4⁺ T cells at the infection site expressing Foxp3. Filarial infection thus favours the development of CD4⁺Foxp3⁺ Treg cells and rapidly biases CD4⁺ T-cell responses towards a regulatory phenotype. Similar increases in Foxp3⁺ Treg cells occurred in both resistant C57BL/6 and susceptible BALB/c mice, indicating that immune failure is not defined solely by CD4⁺Foxp3⁺ Treg-cell recruitment. CD4⁺Foxp3⁺ Teff cells from resistant mice, however, showed augmented levels of activation and *in vivo* proliferation compared with susceptible animals, suggesting that they mount stronger initial CD4⁺ Teff-cell responses or are more resistant to CD4⁺Foxp3⁺ Treg-mediated suppression. In contrast to established infection where two-step treatments are required to break immune-suppression, targeting the initial Treg-cell response alone was sufficient to promote protective immunity. Antibody-mediated depletion of 'natural' CD25⁺Foxp3⁺ Treg cells 7 days prior to infection of susceptible BALB/c mice, while not affecting larval establishment over the first 20 days, resulted in reduced parasite burdens 60 days post-infection and increased parasite-specific cytokine responses. Anti-CD25 treatment also reduced the fecundity of the surviving worms and the incidence of mice developing a patent infection. Thus, the CD4⁺Foxp3⁺ Treg-cell responses that inhibit protective immunity to filarial parasites are initiated very rapidly upon initial infection by the invading L3

larvae and are largely recruited from the natural pool of CD4⁺ Foxp3⁺ Treg cells.

Results

Initial CD4⁺ T-cell responses to *L. sigmodontis* are biased towards Foxp3⁺ Treg

In the *L. sigmodontis* model, CD4⁺Foxp3⁺ Treg cells were previously studied during the chronic, established adult phase (4 wk post-infection) and shown to play a functional role in inhibiting protective immunity against the parasite [23, 50]. To determine whether the CD4⁺Foxp3⁺ Treg-cell response is initiated immediately upon infection by the infective-stage larvae, resistant C57BL/6 and susceptible BALB/c mice were infected s.c. with 25 *L. sigmodontis* L3 parasites and autopsied on days 7 and 12. Following s.c. inoculation, *L. sigmodontis* L3 migrate to the pleural cavity within 3–4 days post-infection and molt to the L4 stage between days 8 and 12. To study T-cell responses during these early stages of infection we sampled the brachial LN (bLN) that drain the s.c. injection site, the thoracic LN (tLN) draining the pleural cavity and the pleural cavity cells representing the site at which infection becomes established.

Increased numbers of total CD4⁺ T cells were seen in both bLN and tLN at days 7- and 12-post-infection (Fig. 1A and B). As would be predicted the bLN showed a larger initial response at day 7 with the tLN cell numbers increasing at day 12 as the larvae migrated from the skin to the pleural cavity. The CD4⁺ T-cell responses in the LN mirrored total cell recruitment (Supporting Information Fig. 1). Despite larvae being present in the pleural

cavity at day 7 (data not shown), total cell numbers were not significantly elevated until 12 days post-infection (Supporting Information Fig. 1C). Interestingly, at day 7 only the resistant C57BL/6 mice showed significantly increased numbers of CD4⁺ T cells in the pleural cavity compared with the naïve controls (Fig. 1C). By day 12 post-infection, however, the CD4⁺ T-cell numbers were equivalently increased in both strains of mice, consistent with previously published work [52].

Infection resulted in a significant expansion of CD4⁺Foxp3⁺ T-cell numbers in the bLN, tLN and pleural cavity (Fig. 1D–F). Despite the increase in total CD4⁺Foxp3⁺ T-cell numbers within the LN, the percentage of LN CD4⁺ T cells expressing Foxp3 did not change (data not shown), signifying that the Treg to Teff cell balance was maintained by equivalent expansion of both CD4⁺ Foxp3[−] Teff and CD4⁺Foxp3⁺ Treg-cell populations. In contrast, within the pleural cavity, the percentage of CD4⁺ T cells expressing Foxp3 in both resistant and susceptible mice was significantly increased upon infection (Fig. 2A and B), indicating that infection favours a CD4⁺Foxp3⁺ Treg-cell response over a CD4⁺Foxp3[−] Teff response. Thus, at the infection site, the local CD4⁺ T-cell response to *L. sigmodontis* infection becomes rapidly biased towards a Foxp3⁺ Treg phenotype.

The majority of CD4⁺Foxp3⁺ Treg cells constitutively express CD25; however, a proportion are known to be CD25[−]. It was therefore important to test whether infection expanded both CD25⁺ and CD25[−] Treg cells. The expansion in the CD4⁺Foxp3⁺ population occurred within both the CD25⁺ and CD25[−] T-cell populations (Fig. 2C), with the proportions of CD25[−] and CD25⁺ cells expressing Foxp3 remaining constant (data not shown). Importantly, the percentage of CD4⁺CD25⁺ T cells expressing Foxp3 at days 7 and 12 post-infection remained equivalent to

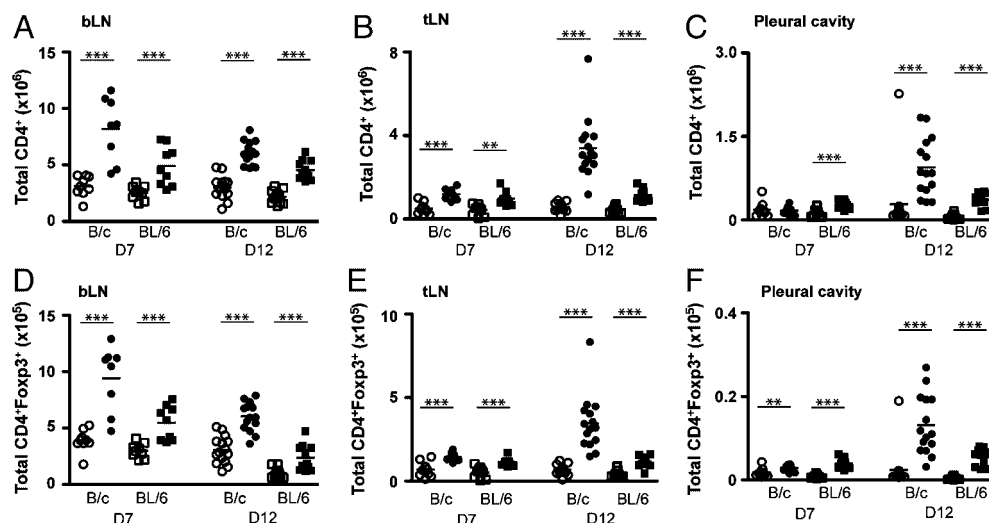


Figure 1. Recruitment of CD4⁺ T cells and CD4⁺Foxp3⁺ Treg cells to the draining LN and pleural cavity during *L. sigmodontis* infection. *L. sigmodontis* infected (closed symbols) and naïve (open symbols) BALB/c (circles) and C57BL/6 (squares) mice were autopsied on days 7 and 12 post-infection. Total CD4⁺ T cells (A–C) and total CD4⁺Foxp3⁺ Treg (D–F), within the bLN (A and D), tLN (B and E), and pleural cavity (C and F) were counted. Symbols represent individual animals, and lines represent mean values. Graphs show pooled data from two experiments for all time points and strains, except BALB/c D12 data pooled from three experiments. ***p* < 0.01, ANOVA, ****p* < 0.001, ANOVA, based on combined data from experiments depicted.

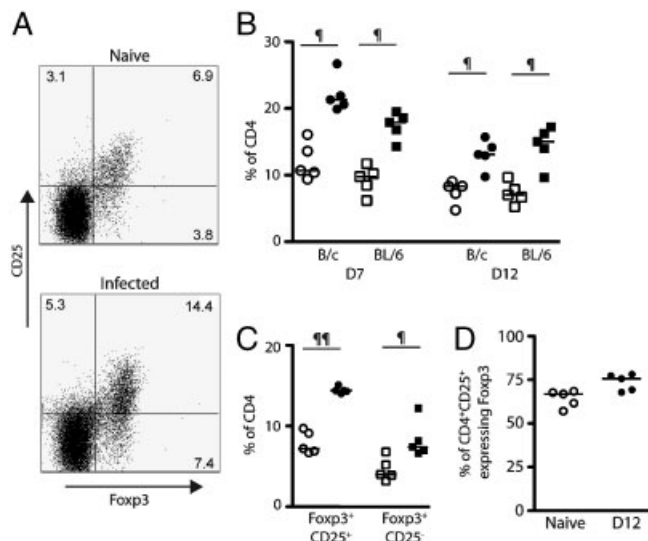


Figure 2. *L. sigmodontis* infection increases the percentage of CD4⁺ Foxp3⁺ T cells within the pleural cavity. Cells were isolated from the pleural cavity of *L. sigmodontis* infected and naïve BALB/c and C57BL/6 mice on days 7 and 12 post-infection. One representative experiment is shown of at least two independent repeats for each time point/strain. Symbols represent individual animals and lines represent median values. (A) FC plots from representative BALB/c mice showing expression of Foxp3 and CD25 at D7. (B) Percentage of pleCD4⁺ T cells from naïve (open symbols) and infected (closed symbols) BALB/c (circles) and C57BL/6 (squares) mice expressing Foxp3. (C) Percentage of Foxp3⁺CD25⁺ (circles) and Foxp3⁺CD25[−] (squares) T cells within the pleCD4⁺ T-cell population of naïve (open symbols) and infected (closed symbols) BALB/c mice 7 days post-infection. (D) Percentage of pleCD4⁺ CD25⁺ T cells from naïve (open symbols) and infected (closed symbols) BALB/c mice expressing Foxp3 12 days post-infection. **p*<0.05 (MWW), ***p*<0.01 (MWW).

naïve animals, demonstrating that at these time points CD25 is still an effective alternative marker for identifying Foxp3⁺ T cells and that the CD4⁺CD25⁺ T-cell population has not been diluted out by CD4⁺CD25[−]Foxp3[−] Teff cells (Fig. 2D).

Filarial infection induces *in vivo* proliferation of Foxp3⁺ Treg cells

The expansion in the CD4⁺Foxp3⁺ Treg-cell population during infection could reflect enhanced proliferation of these cells *in vivo*. To address this we administered BrdU to C57BL/6 and BALB/c mice from days 3 to 7 to label dividing cells *in vivo* and autopsied them on day 7. The percentage of pleural cavity (ple)CD4⁺Foxp3⁺ Treg cells that took up BrdU significantly increased upon infection in both susceptible BALB/c and resistant C57BL/6 hosts (Fig. 3A and B), indicating that infection is actively stimulating CD4⁺Foxp3⁺ Treg cells to divide. Thus, increased proliferation of CD4⁺Foxp3⁺ Treg cells at least partly explains their expansion upon infection, although other factors such as recruitment or increased survival may also be involved.

L. sigmodontis infection also stimulates a Teff-cell response in both mouse strains, as shown by the augmentation in pleCD4⁺

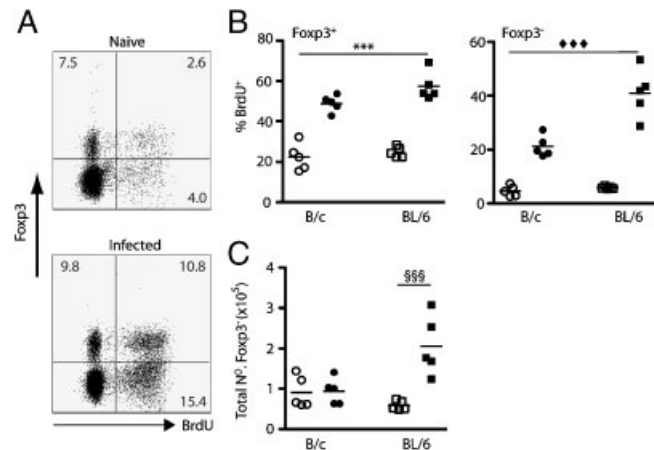


Figure 3. *L. sigmodontis* infection results in increased proliferation of CD4⁺Foxp3⁺ T cells *in vivo*. Pleural cavity cells were isolated from *L. sigmodontis* infected and naïve BALB/c and C57BL/6 mice 7 days post-infection. Panels show one representative experiment of two independent repeats. (A) FC plots from representative naïve and infected BALB/c mice showing expression of Foxp3 and uptake of BrdU by pleCD4⁺ T cells. (B) Percentage of pleCD4⁺Foxp3⁺ and pleCD4⁺Foxp3[−] T cells from naïve (open symbols) and infected (closed symbols) BALB/c (circles) and C57BL/6 (squares) mice positive for BrdU uptake 7 days post-infection. Symbols represent individual animals and lines represent mean values. The effects of infection status and strain on BrdU uptake was analysed using 2-way ANOVA. ***Significant difference in BrdU uptake between naïve and infected mice with no difference between strains, *p*<0.001. ****Significant difference in BrdU uptake between naïve and infected mice (*p*<0.001) and between BALB/c and C57BL/6 mice (*p*<0.001). (C) Total number of CD4⁺Foxp3[−] T cells isolated from the pleural cavity of naïve (open symbols) and infected (closed symbols) BALB/c (circles) and C57BL/6 (squares) mice 7 days post-infection. Symbols represent individual animals and lines represent mean values. \$\$\$*p*<0.001, unpaired t-test.

Foxp3[−] Teff cells incorporating BrdU following infection (Fig. 3B). The CD4⁺Foxp3[−] Teff response in the susceptible BALB/c mice was, however, weaker than that in the resistant C57BL/6 mice based on the percentage of CD4⁺Foxp3[−] Teff cells taking up BrdU. In accord with the muted levels of CD4⁺Foxp3[−] Teff-cell proliferation in the BALB/c mice, when the total numbers of CD4⁺Foxp3[−] Teff cells in the pleural cavity were calculated at day 7 post-infection, significant expansion of the CD4⁺Foxp3[−] population was evident only in the resistant C57BL/6 mice (Fig. 3C).

CD4⁺Foxp3⁺ Treg from infected animals show increased levels of activation

We next inquired whether CD4⁺Foxp3⁺ Treg and CD4⁺Foxp3[−] Teff cells displayed other markers of activation during infection, in addition to heightened proliferation. The percentage of both Foxp3⁺ and Foxp3[−] cells expressing the co-stimulatory molecule ICOS in the pleural cavity (Fig. 4A and E) and bLN (data not shown) of BALB/c mice was significantly increased at 7 days post-infection. Expression of the co-inhibitory receptor PD-1 was also up-regulated among pleFoxp3⁺ and pleFoxp3[−] populations from

infected BALB/c mice demonstrated by significant increases in frequency (Fig. 4B and F) and intensity of expression (Fig. 4G). Similarly, a significant accretion of both the percentages of pleCD4⁺CD25⁺CTLA-4⁺ and pleCD4⁺CD25⁺CTLA-4⁺ T cells was seen in BALB/c mice by 12 days post-infection (Fig. 4C and H). At this stage of infection CD25 remains an effective surrogate marker for Foxp3⁺ Treg cells (Fig. 2D).

In comparing T-cell expression patterns between resistant and susceptible genotype mice, a broadly similar profile was observed except in the case of the co-stimulatory molecule GITR. While GITR expression within pleCD4⁺CD25⁺ Treg cells was up-regulated in both resistant and susceptible strains of mice alike, CD4⁺CD25⁺ Teff cells showed significantly higher GITR levels only in resistant C57BL/6 mice (Fig. 4D, I and J). Thus, although CD4⁺Foxp3⁺ Treg-cell responses are broadly equivalent in the two strains, the susceptible BALB/c mouse is characterized by diminished effector cell proliferation and activation within the context of lower total T-cell numbers after infection.

L. sigmodontis expanded CD4⁺CD25⁺ Treg cells are functionally suppressive

To verify that the *L. sigmodontis* expanded CD4⁺Foxp3⁺ Treg-cell population is functionally suppressive, we performed an *in vitro* suppression assay. At day 12 post-infection CD25 is still an effective marker for CD4⁺Foxp3⁺ Treg cells (Fig. 2D), and in the absence of a direct marker for Foxp3 we purified BALB/c CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells from the pleural cavity 12 days post-infection, and from the spleens of naïve mice. The ability of each population of CD4⁺CD25⁺ T cells to suppress the anti-CD3-stimulated proliferation of the CD4⁺CD25⁺ T cells was then compared. As expected the CD4⁺CD25⁺ T cells from the spleens of naïve mice were able to inhibit the proliferation of the naïve CD4⁺CD25⁺ T cells (Fig. 5). Similarly, pleCD4⁺CD25⁺ T cells of infected mice blocked proliferation of pleCD4⁺CD25⁺ T cells, demonstrating that CD4⁺CD25⁺ phenotype cells from infected mice act in a regulatory manner.

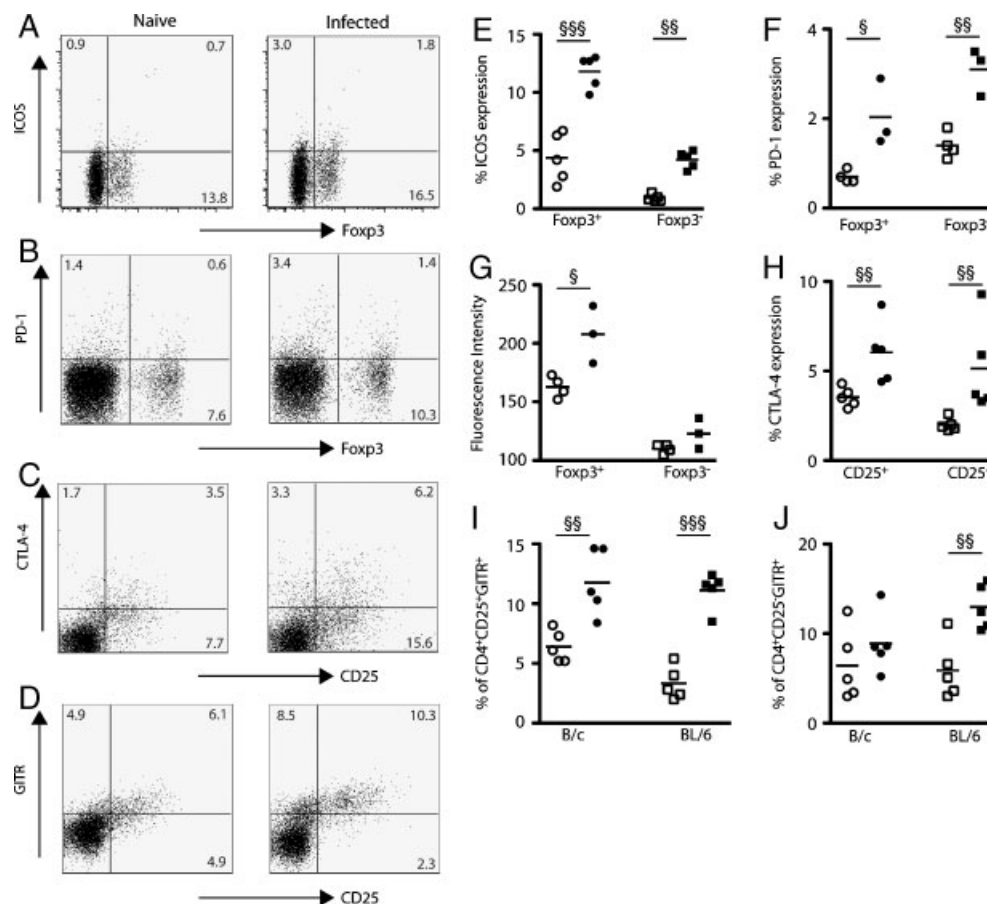


Figure 4. Activation phenotype of CD4⁺Foxp3⁺ Treg and CD4⁺Foxp3⁺ Teff cells during filarial infection. Pleural cavity cells were isolated from naïve (open symbols) and infected (closed symbols) BALB/c and C57BL/6 mice and the expression of ICOS (A and E), PD-1 (B, F and G), CTLA-4 (C and H) and GITR (D, I and J) by CD4⁺ Treg cells (Foxp3⁺ or CD25⁺) and CD4⁺ Teff cells (Foxp3⁺ or CD25⁺) was assessed by FC. Results for ICOS and PD-1 are taken 7 days post-infection. Results for CTLA-4 and GITR are taken 12 days post-infection. FC plots show representative stains from BALB/c mice. Graphs show one representative experiment of two independent repeats. Symbols represent individual animals, and lines represent mean values. §*p*<0.05, §§*p*<0.01, §§§*p*<0.001 (unpaired t-test).

PC61 anti-CD25 treatment effectively depletes CD4⁺ Foxp3⁺ Treg cells in vivo

The rapid increase in CD4⁺Foxp3⁺ Treg during the early stages of *L. sigmodontis* infection led us to hypothesize that natural CD4⁺Foxp3⁺ Treg cells inhibit the development of protective immunity. To test this we used an anti-CD25 (clone PC61)

antibody to deplete susceptible BALB/c mice of CD4⁺CD25⁺ Treg cells. PC61 was administered 7 days prior to *L. sigmodontis* infection to target pre-existing 'natural' CD4⁺ Foxp3⁺ Treg cells and to minimize effects PC61 may have on any cells that up-regulate CD25 upon infection.

Recently, the *in vivo* effectiveness of the two anti-CD25 mAb clones, PC61 and 7D4, in depleting Treg cells has been subject to controversy [53–56]. In particular, 7D4 has been shown to down-regulate CD25 expression on CD4⁺Foxp3⁺ Treg cells without causing depletion [54, 55]. While PC61 does clearly reduce the numbers of CD4⁺Foxp3⁺ Treg cells, depletion is not complete and its effectiveness varies between studies [54, 57]. We therefore wanted to confirm the efficacy of PC61 treatment in depleting CD4⁺ Foxp3⁺ Treg cells in our model. PBL were taken 6 days post-PC61 treatment (1 day prior to infection) and stained for CD4, CD25 and Foxp3. PC61 anti-CD25 treatment reduced CD4⁺CD25⁺ cell levels by 85% (from 5.9 ± 0.6 to $0.9 \pm 0.1\%$), and levels of CD4⁺Foxp3⁺ cells by 72% (from 9.7 ± 1.0 to $2.7 \pm 0.6\%$). CD25 depletion had little effect on the CD4⁺Foxp3⁺CD25⁺ Treg-cell population, which largely accounted for residual CD4⁺Foxp3⁺ Treg-cell numbers after PC61 treatment (data not shown).

The effects of PC61 pre-treatment on the initial Treg-cell response to infection were assessed at day 12 post-infection (19 days post-depletion). PC61 treated naïve mice had an 85%

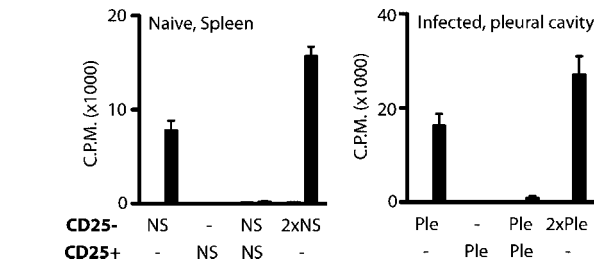


Figure 5. *L. sigmodontis* recruited Treg are suppressive *in vitro*. Pooled CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells were purified from the pleural cavity of *L. sigmodontis*-infected BALB/c mice 12 days post-infection (Ple), and from the spleens of naïve BALB/c mice (NS). The CD4⁺CD25⁺ and CD4⁺CD25⁺ T-cell populations were cultured alone or mixed in a 1:1 ratio and their proliferation in response to medium alone (open bars) or anti-CD3 (closed bars) measured. Figure shows mean and standard deviation of cultures in triplicate.

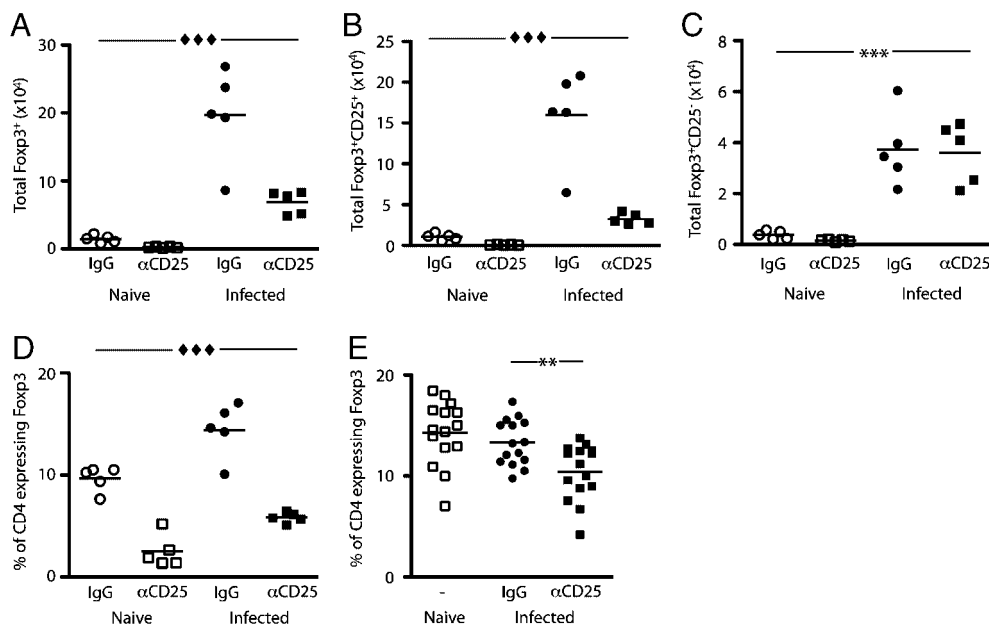


Figure 6. Anti-CD25 treatment using clone PC61 effectively depletes CD4⁺Foxp3⁺ Treg cells in naïve and *L. sigmodontis*-infected mice. BALB/c mice were treated with an anti-CD25-depleting antibody or rat IgG control Ab 7 days prior to infection. Pleural cavity cells were isolated 12 days post-infection and stained for CD4, CD25 and Foxp3. Panels A–D show one representative experiment of 2. Symbols represent individual animals and lines represent mean values. Total number of pleCD4⁺Foxp3⁺ (A), pleCD4⁺Foxp3⁺CD25⁺ (B) and pleCD4⁺Foxp3⁺CD25⁺ (C) T cells of naïve (open symbols) and infected (closed symbols) BALB/c mice that had been pre-CD25 depleted (squares) or treated with control rat IgG (circles). (D) Percentage of pleCD4⁺ T cells expressing Foxp3 in naïve (open symbols) and infected (closed symbols) mice following CD25 depletion (squares) and IgG control treatment (circles). In panels A–D the effects of infection and treatment were analysed using two-way ANOVA. ***Significant difference due to treatment ($p < 0.001$) and infection ($p < 0.001$). ***Significant difference due to infection status ($p < 0.001$), but no significant difference caused by treatment. (E) Percentage of CD4⁺ T cells expressing Foxp3 in the tLN of naïve untreated (open squares), infected IgG-treated (closed circles), or infected and CD25 pre-depleted BALB/c mice (closed squares) 60 days post-infection. Panel shows pooled data from three independent experiments. ** $p < 0.01$, two-way ANOVA based on combined data from three experiments.

reduction in the total numbers of CD4⁺Foxp3⁺ Treg cells in the pleural cavity (Fig. 6A). They also had an 81% reduction in the total numbers of CD4⁺Foxp3⁺ T cells in the bLN and a 54% reduction in the tLN (data not shown). Thus, there were still significant reductions in CD4⁺Foxp3⁺ T-cell numbers in naïve mice 19 days post-PC61 treatment. In common with anti-CD25 treatments during *Plasmodium yoelii* infection [54], subsequent infection of PC61 treated mice with *L. sigmodontis* resulted in a faster repopulation of CD4⁺Foxp3⁺ cells compared with the naïve controls (Fig. 6A). It was, however, notable that the total numbers of CD4⁺Foxp3⁺T cells in the pleural cavity of PC61 treated and infected BALB/c mice were still diminished by 65% compared with the IgG-treated infected controls (Fig. 6A), and that there were reductions of 71 and 62% in the tLN and bLN, respectively (data not shown).

Relating the reduction in CD4⁺Foxp3⁺ T-cell numbers to CD25 expression showed that all of the depletion occurred in the CD4⁺Foxp3⁺CD25⁺ population (Fig. 6B and C). Within the pleural cavity, PC61 treatment reduced the total numbers of CD4⁺Foxp3⁺CD25⁺ T cells by 94% in naïve mice, and by 80% in mice subsequently infected with *L. sigmodontis* (Fig. 6B). Within the tLN and bLN CD4⁺Foxp3⁺CD25⁺ Treg cells were reduced by 73 and 86%, respectively, in naïve mice, and by 79 and 73%, respectively, in mice that were subsequently infected (data not shown). In contrast, PC61 treatment had little effect on the levels of CD4⁺Foxp3⁺CD25[−] Treg cells, which expanded

normally in response to infection in the PC61-treated mice (Fig. 6C). Despite the lack of effect on the CD25[−]Foxp3⁺ Treg cells, PC61-treatment prevented the preferential expansion in CD4⁺Foxp3⁺ Treg cells upon infection with the percentage of pleCD4⁺ T cells expressing Foxp3 in CD25-depleted, infected mice being significantly less than that of both the naïve and infected IgG control mice (Fig. 6D). Strikingly, the percentage of CD4⁺ T cells expressing Foxp3 in the tLN of anti-CD25-depleted and infected BALB/c mice was also significantly decreased 60 days post-infection compared with the infected IgG controls indicating a long-term effect of PC61 treatment (Fig. 6E).

Thus, in our system PC61 anti-CD25 treatment was effective at depleting CD4⁺Foxp3⁺CD25⁺ T cells *in vivo*, resulting in loss of the majority of the CD4⁺Foxp3⁺ T-cell population. Importantly, PC61-treated mice subsequently infected with *L. sigmodontis* had greatly reduced numbers of CD4⁺Foxp3⁺ T cells, and treatment prevented the early infection-induced bias towards CD4⁺Foxp3⁺ Treg cells in the pleural cavity.

CD4⁺Foxp3⁺CD25⁺ natural Treg inhibit protective immunity to *L. sigmodontis*

Following confirmation that PC61 treatment depletes CD4⁺CD25⁺ Foxp3⁺ Treg cells *in vivo* we assessed the effects of depleting CD4⁺CD25⁺ Treg cells 7 days prior to infection on parasite survival and

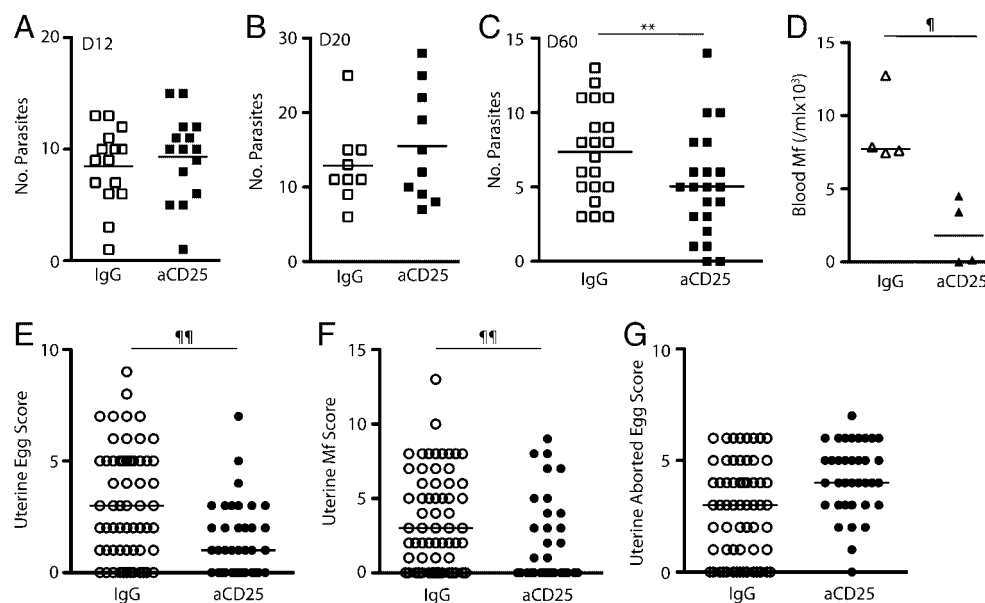


Figure 7. CD4⁺CD25⁺Foxp3⁺ natural Treg inhibit protective immunity to *L. sigmodontis* infection. BALB/c mice were treated with a depleting anti-CD25 Ab (closed symbols) or rat IgG (open symbols) 7 days prior to *L. sigmodontis* infection. Parasite burdens were assessed on days 12 (A), 20 (B) and 60 (C) days post-infection. For days 12 and 60 figures show pooled results from three and four independent experiments, respectively, using infective doses of 25L3. The results at day 20 show two independent experiments using infective doses of 25 and 40L3. Symbols represent individual animals, and lines represent mean values. **Significant difference in parasite recoveries between treatment groups ($p < 0.01$, ANOVA, based on combined data from four experiments). (D) Number of Mf/ml in the blood of microfilaraemic mice from experiment 4. Symbols represent individual animals, and lines represent median values. *Significant different between treatment groups ($p < 0.05$, MWW). (E–G) Semi-quantitative scores for the number of healthy eggs (E), Mf (F) and aborted eggs (G) in the uterus of female parasites recovered 60 days post-infection. Panels show data pooled from three independent experiments. Symbols represent individual female parasites, and lines represent median values. **Significant difference between treatment groups ($p < 0.01$, MWW).

fecundity. No immediate effect on parasite recoveries was seen in CD25-depleted BALB/c mice at days 12 (Fig. 7A) or 20 (Fig. 7B) post-infection. Parasite growth, defined by parasite length, was also unaffected at these time points (data not shown).

Although CD4⁺CD25⁺ Treg cells, which form the majority of the Foxp3⁺ T cells, do not appear to play a unique role in initial larval survival and establishment, pre-CD25 depletion did affect the subsequent adult stage of infection and reproducibly inhibited the ability of the parasites to establish a fully patent infection, albeit with a different efficacy of treatment between experiments. By 60 days post-infection, parasite recoveries were significantly reduced by 31% across four experiments (Fig. 7C). Despite decreased parasite numbers, treatment had a limited effect on parasite growth, with a significant reduction in length seen in female parasites in only one of the four experiments (data not shown).

A more pervasive effect of pre-CD25 depletion was observed as a decrease in the number of mice that developed patent infections, as defined by the presence of Mf circulating in the blood stream. The incidence of patency was reduced following treatment in two out of three experiments and in one of these experiments was completely prevented (Table 1). We should note that the assessment of patency is limited to three of the four experiments because the IgG control animals in one experiment failed to develop blood Mf. In total, and as previously recorded [58], 69% of the IgG control mice developed a patent infection, compared with 33% in the pre-CD25 depleted mice. Even in the one experiment in which the incidence of patency was unaffected by anti-CD25 treatment (Experiment 4), the concentration of Mf circulating in the bloodstream was significantly reduced (Fig. 7D).

To determine whether the reduced incidence and levels of Mf in the blood of treated mice was due to impaired female fecundity or increased killing of the Mf once released into the host we semi-quantitatively scored the numbers of healthy eggs, Mf, and aborted eggs within the uteri of the surviving female parasites. Female parasites recovered from the anti-CD25 depleted mice had significantly fewer healthy eggs (Fig. 7E) and Mf (Fig. 7F) in their uteri compared with female parasites recovered from IgG treated control mice. The uteri of female parasites from anti-CD25 treated mice also tended to contain higher numbers of

aborted eggs than did the uteri of female parasites from IgG-treated control mice (Fig. 7G). Thus, CD4⁺CD25⁺ natural Treg inhibit protective immunity towards filarial parasites, and their depletion results in a significant impairment of parasite survival and fecundity.

Parasite killing is associated with an enhanced immune response

We were interested to determine whether the increased parasite killing caused by depletion of CD25⁺ Treg cells was associated with enhanced immune responses. Depletion of CD25⁺ Treg cells prior to infection of susceptible BALB/c mice resulted in an up-regulation of GITR expression by CD4⁺CD25⁺ Teff cells at days 12 (Fig. 8A) and 20 (Fig. 8B) post-infection, recapitulating the Teff phenotype of the resistant C57BL/6 mouse (Fig. 4J). Thus, natural CD25⁺Foxp3⁺ Treg cells appear to inhibit Teff-cell activation during the initial stage of infection. CD25⁺ T-cell depletion did not, however, translate to early increases in total CD4⁺ T-cell numbers at the infection site or draining LN, or to antigen-specific cytokine production (IL-4, IFN- γ , IL-5 and IL-10) at days 12 or 20 post-infection (data not shown). When cytokine production by tLN cells was assessed 60 days post-infection, at the time when parasite killing was enhanced (Fig. 7), the pre-infection anti-CD25 depleted mice were found to produce significantly increased levels of IL-4, IL-5, IL-13 and IL-10 in response to *L. sigmodontis* antigen (Fig. 8C–F). Anti-CD25 treatment did not result in increased antigen-specific proliferation *in vitro* at any time point (data not shown). Thus, depletion of CD25⁺ Treg prior to infection does result in enhanced antigen-specific immunity; however, except for changes in GITR expression at days 12 and 20 this was not manifest until the adult stages of infection, at which time deficits in parasite viability and fecundity became evident.

Discussion

The involvement of Treg cells during chronic stages of human filarial infections is increasingly evident [10, 19–21], supporting the hypothesis that this T-cell phenotype contributes to the immune suppression so characteristic of helminth infections [5–7]. We have shown that CD4⁺Foxp3⁺CD25⁺ Treg cells are active in susceptible BALB/c mice during the adult stages of *L. sigmodontis* infection and inhibit protective immune responses resulting in enhanced parasite survival [23, 50]. It is therefore important to understand the origins of Foxp3⁺ Treg-cell responses, whether they are generated from the initial point of infection by infective-stage L3 larvae or arise subsequently as infection reaches stable chronicity, as well as whether they are recruited from the pool of 'natural' Foxp3⁺Treg or induced from naïve T cells. We now demonstrate that the CD4⁺Foxp3⁺ Treg-cell response is induced by the infective L3 larvae immediately

Table 1. Incidence and percentage of anti-CD25-depleted and IgG-treated mice developing blood microfilaraemia 60 days post *L. sigmodontis* infection

	Treatment			
	IgG		Anti-CD25	
	Incidence	%	Incidence	%
Exp. 1	4/5	80	0/4	0
Exp. 3	3/5	60	1/5	20
Exp. 4	4/6	67	4/6	67
Combined	11/16	69	5/15	33

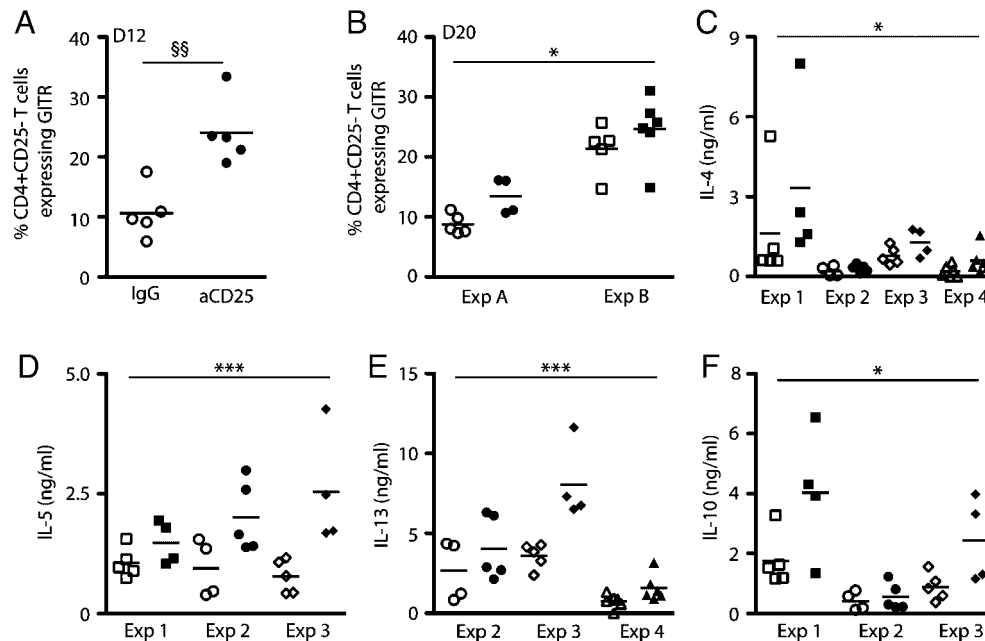


Figure 8. CD4⁺CD25⁺Foxp3⁺ Treg inhibit antigen-specific immunity. BALB/c mice were treated with a depleting anti-CD25 mAb (closed symbols) or rat IgG (open symbols) 7 days prior to *L. sigmodontis* infection. Symbols represent individual animals and lines represent mean values. (A) The percentage of pleCD4⁺CD25⁺ T cells expressing GITR at day 12 post-infection. (B) Percentage of pleCD4⁺CD25⁺ T cells expressing GITR at day 20 post-infection. Figure shows results from two individual experiments (represented by different symbols). (C–F) *In vitro* production of IL-4 (C), IL-5 (D), IL-13 (E) and IL-10 (F) in response to stimulation with LsAg by tLN cells isolated 60 days post-infection. Background cytokine production by medium controls has been subtracted. Panels show results from three or four independent experiments. §§*p*<0.01, *t*-test. *Significant difference between treatment groups (*p*<0.05, ANOVA). ***Significant difference between treatment groups (*p*<0.001, ANOVA). Two-way ANOVA was performed across combined data from experiments depicted in the individual panels.

upon infection. Recruitment of natural CD4⁺Foxp3⁺CD25⁺ Treg cells forms a major component of this response, and depleting natural CD4⁺CD25⁺ Treg cells prior to infection enhances parasite killing.

Initial exposure to *L. sigmodontis* L3 larvae preferentially drove a CD4⁺Foxp3⁺ Treg-cell response, skewing the CD4⁺ T-cell response at the infection site (pleural cavity) towards a Foxp3⁺ Treg-cell phenotype within the first 7 days of infection. This Treg-cell bias was distinguished by increased *in vivo* proliferation and total numbers of CD4⁺Foxp3⁺ Treg cells in the pleural cavity and draining LN, and most notably by an increased percentage of pleCD4⁺ T cells expressing Foxp3. During *Leishmania major* infection, a similar imbalance in the ratio of CD4⁺Foxp3⁺ to CD4⁺Foxp3[−] T cells results in a failure of sterilising immunity and the persistence of long-term low-level infections [59, 60]. Long-lived helminth infections are also associated with outgrowth of CD4⁺Foxp3⁺ Treg cells in draining LN during *Heligmosomoides polygyrus* infection [25] and around muscle-encysted *Trichinella spiralis* larvae [24]. Thus, we hypothesized that the rapid recruitment of CD4⁺Foxp3⁺ Treg cells during the initial stages of *L. sigmodontis* infection impairs the generation of CD4⁺ T-effector cell responses and so inhibits parasite killing.

The role of CD4⁺Foxp3⁺ Treg cells in promoting the survival of *L. sigmodontis* was confirmed using an anti-CD25 antibody to deplete CD4⁺CD25⁺Foxp3⁺ Treg cells from BALB/c mice prior to

infection. This strain is fully susceptible to *L. sigmodontis* infection, reaching patency (bloodstream Mf) around 50 days post-infection [48, 49]. Although treatment did not notably affect early larval establishment, anti-CD25-depleted BALB/c mice showed reduced parasite burdens 60 days post-infection. The efficacy of treatment was further confirmed when the surviving female parasites from anti-CD25-treated mice were found to have reduced fecundity. This resulted in a lower proportion of anti-CD25-depleted mice developing fully patent infections. The ability to increase killing of *L. sigmodontis* by depleting CD25⁺ cells prior to infection infers that recruitment of natural CD4⁺Foxp3⁺ Treg forms an important component of the early Treg-cell response to filarial larvae.

The promotion of an anti-fecundity effect highlights a consistent and significant aspect of immunity to helminths. For example, in *Schistosoma haematobium* infections, egg output declines in a T-cell-dependent manner [61], while fecundity of *H. polygyrus* is raised following anti-CD4 depletion [62]. IL-4 is an important component in the regulation of *H. polygyrus* egg production [63] and is also required to control microfilarial output in *Brugia pahangi* [64] and *L. sigmodontis* [65]. Anti-fecundity effects have been replicated by passive transfer of immune serum [66] and are also observed in a number of recombinant anti-helminth vaccination trials [67, 68]. Reduction of parasite fertility may therefore be a sensitive, and quantitative, measure of host immunity, and

one which becomes evident even where the immune system is not yet able to kill the adult worm. Depletion of CD25⁺ Treg may remove a major restraint on anti-fecundity effects, resulting in uplift in reactivity and a significant loss of parasite reproductive capacity.

Despite the clear attenuating effects of depleting CD25⁺ Treg prior to infection, it is notable that over the first 20 days the larval stages were unaffected, and even at day 60 the clearance of adults was incomplete. Several factors may contribute to this finding. One possibility is that while CD4⁺Foxp3⁺CD25⁺ cells form the majority of the CD4⁺Foxp3⁺ population, anti-CD25 treatment had very little effect on the CD4⁺Foxp3⁺CD25⁺ Treg cells, which expanded normally upon infection of anti-CD25-depleted mice. These remaining CD4⁺Foxp3⁺CD25⁺ cells may have been sufficient to still impact on immunity. Alternatively, *L. sigmodontis* infection may also stimulate the production of inducible CD4⁺Foxp3⁺ Treg cells that act in concert with the 'natural' CD4⁺Foxp3⁺ Treg. Induced CD4⁺Foxp3⁺ Treg cells would not have been targeted by the anti-CD25 treatment given prior to infection and thus may still have been able to inhibit the CD4⁺ Teff cells. The increases in CD4⁺Foxp3⁺ Treg cells in the anti-CD25 treated mice, however, were slight and if CD4⁺Foxp3⁺ Treg cells play a major role in larval survival over the first 20 days it is likely that an effect would have been seen following treatment. An explanation that we favour is that filarial infections use many different means to inhibit host immunity including targeting DC [69, 70], recruiting suppressive macrophages [71, 72] and releasing immunomodulatory molecules [47, 73]. Thus, we suggest that the recruitment of natural CD4⁺Foxp3⁺ Treg represents one of numerous levels of immune down-regulation and so their depletion only partially restores protection.

The promotion of protective immunity though a single treatment with anti-CD25 prior to infection contrasts with the ineffectiveness of solely administered anti-CD25 during the established adult stage of infection, 28 days post-infection [50]. During adult *L. sigmodontis* infection, alongside active CD4⁺Foxp3⁺ Treg-cell responses, CD4⁺ Teff cells develop an antigen-specific hypo-responsive phenotype resulting in two layers of T-cell regulation [23, 50]. Evidence for interplay between Foxp3⁺ Treg cells and T-cell anergy is also found in chronic human filarial infections [10]. Once established, *L. sigmodontis* infection can only be curtailed with anti-CD25 treatment when combined with boosting the CD4⁺ Teff-cell response by providing co-stimulation through an agonistic anti-GITR mAb [50] or blocking inhibitory signals through CTLA-4 [23]. The success of single pre-infection anti-CD25 treatments may reflect the fact that Treg cells were depleted before CD4⁺ Teff cells develop a hypo-responsive phenotype, and thus the additional anti-GITR or anti-CTLA-4 treatments were not required. Alternatively, CD4⁺Foxp3⁺ Treg cells may induce CD4⁺ Teff cells to become hypo-responsive and thus their depletion prior to infection may prevent the development of T-cell hypo-responsiveness. This indicates that the timing of anti-CD25 treatment is critical in determining its effectiveness and that infection

becomes more difficult to treat once immune-regulatory pathways have established.

The implication that other immune down-regulatory mechanisms are acting alongside Treg cells to permit larval establishment fits with the observation that both resistant C57BL/6 and susceptible BALB/c mice mounted a similar initial CD4⁺Foxp3⁺ Treg-cell response. Thus, recruitment of Treg *per se* does not explain the difference between the resistant and susceptible phenotypes. Instead the strain differences observed lay in the CD4⁺ Teff-cell population. Within the first 12 days, CD4⁺CD25⁺ Teff cells from resistant C57BL/6 mice up-regulated expression of GITR in response to infection, whereas those from the susceptible BALB/c mice did not. The up-regulation of GITR in C57BL/6 mice was also associated with greater *in vivo* proliferation of the CD4⁺Foxp3⁺ Teff cells. This suggests that the resistant C57BL/6 mice are more effective at priming their CD4⁺ T-cell response than the susceptible BALB/c mice or are more resilient to the initial suppressive effects of the filarial L3 parasites.

The lack of effect of anti-CD25 depletion on antigen-specific cytokine responses within the first 20 days of infection is interesting given that treatment promoted parasite killing. Depletion of CD25⁺ cells prior to infection with *B. pahangi* does result in an increased Th2 response 12 days post-infection, indicating that natural Treg can inhibit early Th2 responses to filarial parasites [74]. Increased *L. sigmodontis*-specific Th2 responses were seen in anti-CD25-depleted BALB/c mice 60 days post-infection (67 days post-depletion), and so in *L. sigmodontis* infection the immunological effects of the CD25 depletion take time to become apparent. One change in T-cell phenotype that did occur early was in the expression of the co-stimulatory molecule GITR by CD4⁺ Teff cells, which increased upon infection in the anti-CD25-depleted BALB/c mice. This indicates that in the absence of CD4⁺CD25⁺Foxp3⁺ Treg the BALB/c CD4⁺ Teff-cell response is primed more effectively, recapitulating the resistant C57BL/6 phenotype. If multiple mechanisms are involved in down-regulating host immunity during infection, then these initial effects of the anti-CD25 depletion on T-cell priming may be masked.

T-cell co-stimulatory signals through GITR are therefore linked with resistance to filarial parasites in the larval as well as the adult stages of *L. sigmodontis* infection. GITR co-stimulation has been shown to render CD4⁺ Teff cells resistant to suppression by CD4⁺Foxp3⁺ Treg [75–77]; thus early up-regulation of GITR by C57BL/6 mice may assist their Teff cells in overcoming increased CD4⁺Foxp3⁺ Treg-cell activity leading to their resistant phenotype. In contrast, by failing to up-regulate GITR the CD4⁺Foxp3⁺ Teff cells of susceptible BALB/c mice may be left vulnerable to the expanded CD4⁺Foxp3⁺ Treg cells. Moreover, the restored GITR expression upon infection of anti-CD25-depleted BALB/c mice indicates that CD4⁺CD25⁺Foxp3⁺ Treg cells themselves inhibit GITR up-regulation. In this scenario, succumbing to Treg-mediated suppression makes Teff cells more susceptible to suppression reinforcing the susceptible phenotype.

Altogether these data indicate that how quickly and effectively Teff responses are initiated will determine the resilience of the

CD4⁺ Teff cells to immune-suppression and may set the basis for immune responses throughout infection. Incorrect priming of T cells leads to an anergic phenotype [78]; thus an initial failure to prime a sufficient effector response may result in hyporesponsiveness later in infection. If the removal of Treg cells results in more robust T-cell priming, while the immunological effects may be initially masked by other regulatory mechanisms, then the T-cell response would be more resistant to parasite-induced immune suppression throughout infection. Although it may still take time for the CD4⁺ Teff response to build to a threshold strong enough to overcome regulation, stronger initial priming would eventually equate to more rapid killing of the parasite. Our data thus suggest that the initial T-cell priming events to the invading L3 larvae are critical in defining immunity to the later stages of infection.

In conclusion, the CD4⁺Foxp3⁺ Treg-cell response that inhibits protective immunity to filarial parasites is driven by the invading L3 larvae immediately upon contact with the host and is recruited largely from the pre-existing pool of natural Treg cells. This results in the initial CD4⁺ T-cell response becoming biased towards a Foxp3⁺ phenotype. In contrast to an established adult *L. sigmodontis* infection, anti-CD25 treatment alone can promote protective immunity when given prior to infection to dampen the initial CD4⁺Foxp3⁺ Treg-cell response. These data indicate that the initial CD4⁺ T-cell response to filarial parasites is important in determining infection outcome and that it is easier to prevent the induction of immune-regulation than to reverse established immune-regulatory networks. This has important implications for control of infection, suggesting that prophylactic treatments will be more effective than post-infection therapies.

Materials and methods

Mice and parasites

Female BALB/c and C57BL/6 mice were used at 6–8 wk of age and maintained in individually ventilated cages. The *L. sigmodontis* life cycle was maintained in gerbils using the mite vector *Ornithonyssus bacoti* [49]. Mice were inoculated s.c. on the upper back with 25 infective *L. sigmodontis* L3 larvae. In one experiment a dose of 40 L3 was used and is indicated in the results. Parasites were recovered by pleural lavage and fixed in hot 70% ethanol for morphological analysis [49]. The analysis of fecundity of female *L. sigmodontis* parasites followed the method of Bretonet *al.* [79]. The numbers of Mf, healthy eggs and aborted eggs within the anterior, median and posterior of the uterus were semi-quantitatively scored on scales of 0–5, 0–3 and 0–3, respectively. Each region's scores were summed giving a total possible Mf score of 15 and egg score of 9. To quantify blood Mf, 30 µL of tail blood was collected in FACS lysing solution (Becton-Dickinson). *L. sigmodontis* whole worm antigen (LsAg) was prepared by collecting the

PBS-soluble fraction of homogenized adult male and female worms.

In vivo antibody and BrdU treatments

Mice received 1 mg of anti-CD25 mAb (PC61, in house) or rat IgG (Sigma-Aldrich) 7 days prior to infection. For BrdU treatment, mice received an i.p. injection of 1 mg BrdU (Sigma-Aldrich) in PBS on day 3 post-infection, followed by fresh BrdU in their drinking water at 1 mg/mL each day until autopsy on day 7 post-infection.

Cell purifications and in vitro restimulations

The bLN were taken as a source of LN draining the s.c. injection site, and the parathymic, posterior mediastinal and paravertebral LN (tLN) were pooled as a source of LN draining the thoracic cavity. LN were dissociated and washed prior to being resuspended in RPMI-1640 with 0.5% mouse sera (Caltag-MedSystems), 100 U/mL penicillin–100 µg/mL streptomycin and 2 mM L-glutamine. Pleural cavity cells were isolated by lavage. CD4⁺CD25[−] and CD4⁺CD25⁺ T cells were purified in a two-step process. CD4 T cells were first enriched by negative magnetic selection using primary antibodies against MHC class II (M5/114.15.2, in house), CD8 (53-6.72, in house), B220 (RAB632, in house), CD11b (M1/70, in house), Gr1 (RB6-8C5, BD Biosciences) and F4/80 (A3-1; Caltag-MedSystems), followed by sheep anti-rat IgG magnetic beads (Dyna). Labelled cells were magnetically depleted using a Dynal MPC-1 magnet (Dyna). The cells were then stained for allophycocyanin-conjugated anti-CD4 (RM4-5, BD Biosciences) and phycoerythrin-conjugated anti-CD25 (7D4, Milteny Biotech) and were sorted into CD4⁺CD25⁺ and CD4⁺CD25[−] cell populations using a FACSAria running FACSDiva software (Becton-Dickinson). The CD4⁺CD25[−] T-cell populations were >99% pure, and the purities of CD4⁺CD25⁺ T-cell populations were >97%. Irradiated (30 Gy) splenic APC from naïve mice were added to 96-well round-bottom plates at 1.5×10^5 cells/well. A total of 7.5×10^4 CD4⁺CD25[−] and CD4⁺CD25⁺ T cells were added separately or in a 1:1 ratio. Whole tLN cells were used at 5×10^5 cells/well. Cultures were stimulated with medium alone, 10 µg/mL LsAg, or 0.1 µg/mL anti-CD3 (4C11, in house). Supernatants were sampled at 72 h for cytokine analysis, and 1 µCi/well methyl-[³H]thymidine added for 16 h to measure proliferation.

Antibodies and reagents

Antibody pairs used for cytokine ELISA were as follow: IL-4 (11B11/BVD6-24G2); IL-5 (TRFK5/TRFK4); IL-10 (JES5-2A5/SXC-1); capture anti-IL-13 (38213, R&D systems); and biotinylated polyclonal anti-IL-13 (Peprotech). Recombinant murine IL-4, IL-10, and IL-5 (Sigma-Aldrich), and IL-13 (R&D Systems)

were used as standards. Biotin detection antibodies were used with ExtrAvidin-alkaline phosphatase conjugate (Sigma-Aldrich) and Sigma Fast™ *p*-nitrophenyl phosphate substrate (Sigma-Aldrich). For flow cytometry (FC), non-specific binding was blocked with 4 µg of rat IgG/1 × 10⁶ cells, and the following antibodies were applied: phycoerythrin-conjugated anti-CTLA-4 (UC10-4F10-11), PD-1 (J43), peridinin chlorophyll protein-conjugated streptavidin, allophycocyanin and Alexa700-conjugated anti-CD4 (RM4-5), biotinylated anti-CD25 (7D4), and ICOS (7E.17G9), fluorescein isothiocyanate-conjugated anti-GITR (DTA-1, in house). Staining for Foxp3 was performed using fluorescein isothiocyanate-, phycoerythrin- and allophycocyanin-conjugated anti-Foxp3 (FJK-16s, eBioscience). BrdU staining was performed after the final Foxp3 staining step using fluorescein isothiocyanate-conjugated anti-BrdU with DNase. Staining was compared with the relevant isotype controls to verify specificity. As the majority of CD4⁺ T cells expressed low levels of GITR when compared with the isotype control, FC plots were gated on CD4⁺ GITR^{hi} cells. To measure intracellular CTLA-4, cells were permeabilized with BD Pharmingen's Cytofix/Cytoperm kit, or with the eBioscience Foxp3 permeabilization kit. Flow cytometric acquisition was performed using a FACScalibur running CellQuest Pro software and an LSR 2 running FACSDiva software (BD biosciences). Analysis was performed using Flowjo (Treestar). Reagents were obtained from BD Biosciences unless otherwise stated.

Statistics

Statistical analysis was performed using JMP version 7 (SAS) according to the following strategy. The data were first checked for homogeneity of variance and normality, which are two major assumptions of parametric statistical analysis. If raw data failed to meet the assumptions log₁₀ or square root transformations were applied, and the data were checked again. Whenever the required assumptions were met, parametric analysis of combined data from multiple repeat experiments, or of experiments containing more than two groups, was performed using ANOVA. For normal data from experiments with two groups, unpaired *t*-tests were used to assess treatment effects. When using two-way ANOVA to combine data from multiple experiments, experimental effects were controlled for in the analysis and it was verified that there were no significant qualitative interactions between experimental and treatment effects. If the data set did not meet the required conditions for parametric tests, comparison between groups was performed using the non-parametric unpaired Mann–Whitney–Wilcoxon rank sum test (MWW). For analysis of uterine egg and Mf contents, female parasites recovered from the IgG control and anti-CD25 groups were pooled between experiments. Prior to pooling, it was verified that the uterine scores of the IgG and anti-CD25 groups did not significantly differ among experiments using the non-parametric Kruskal–Wallis test. Figures depict means whenever parametric tests were used, and medians whenever non-parametric tests were used.

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Abbreviations: bLN: brachial LN · FC: flow cytometry · LsAg: *Litomosoides sigmodontis* whole worm antigen · Mf: microfilaria · MWW: Mann–Whitney–Wilcoxon rank sum test · pleCD4⁺T cells: pleural cavity CD4⁺T cells · Teff cell: effector T cell · tLN: thoracic LN

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